

Relationships among Bone Quality, Implant Osseointegration, and Wnt Signaling

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Appendix

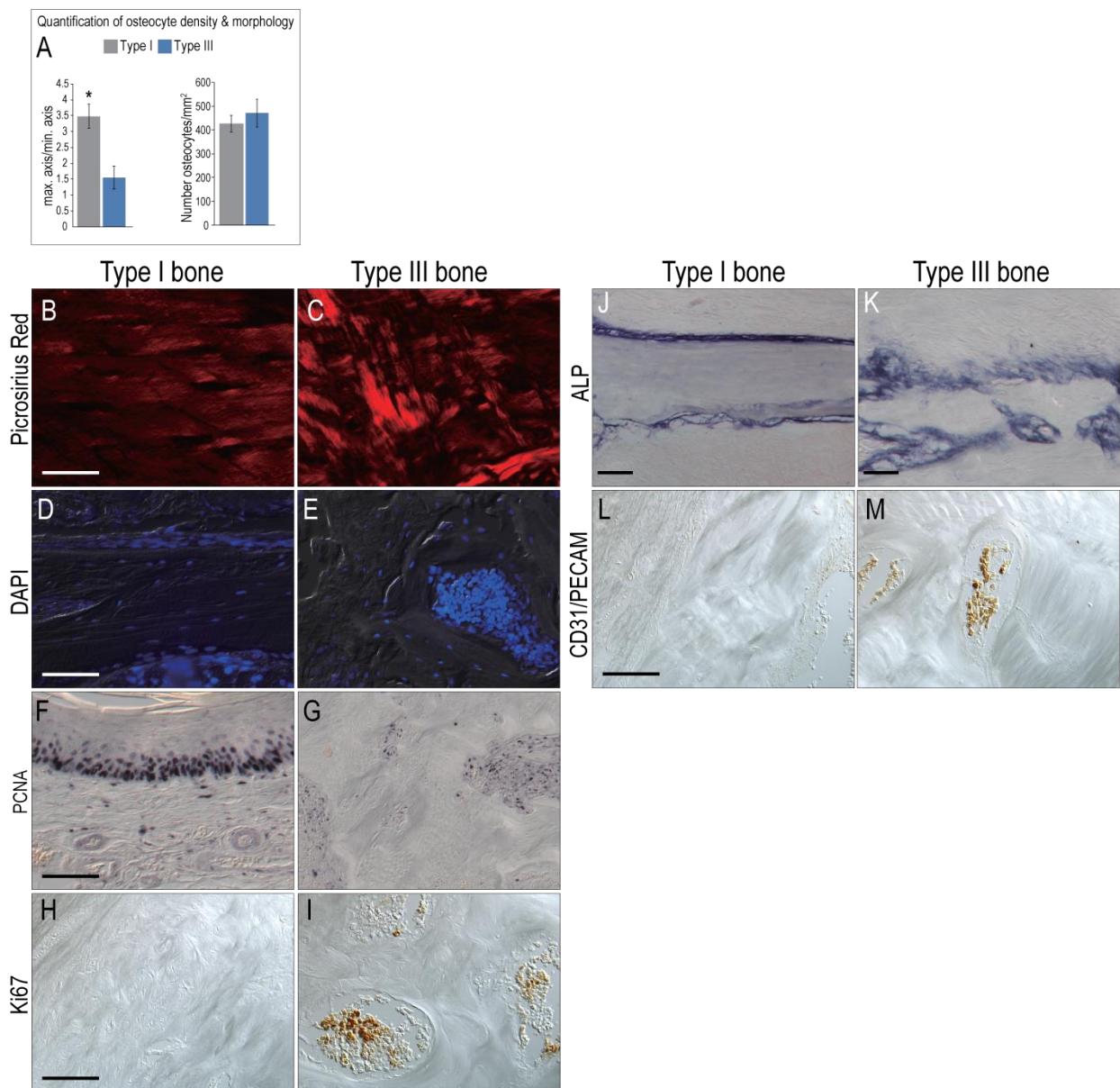
Appendix Table 1. Experimental groups and analyses performed

Bone type	N	M1 extraction	Genotype & age at surgery	Anatomical site	Osteotomy dia. (mm)	Tissue collection	Analyses	Implant	Data shown in Fig.
I	6	no	WT, 12w	Edentulous ridge	-	Skeletally mature adult	Histology, IHC, qRT-PCR, SEM	-	1, SFig. 1
III	6	no	WT, 12w	Alveolar bone	-	Skeletally mature adult	Histology, IHC, qRT-PCR, SEM	-	1, SFig. 1
III	6	yes	WT, 8w	Alveolar bone	-	4	Histology, histomorphometry	-	SFig. 2
III	6	yes	WT, 8w	Alveolar bone	-	7	Histology, histomorphometry	-	SFig. 2
III	6	yes	WT, 8w	Alveolar bone	-	14	Histology, histomorphometry	-	SFig. 2
III	6	yes	WT, 8w	Alveolar bone	-	28	Histology, histomorphometry	-	SFig. 2
III	8	yes	WT, 12w	Healed extraction socket	1.0	2	Histology, IHC, qRT-PCR, histomorphometry	-	2
III	8	yes	WT, 12w	Healed extraction socket	1.0	4	Histology, IHC, qRT-PCR, histomorphometry	-	SFig. 3
III	8	yes	WT, 12w	Healed extraction socket	1.0	7	Histology, IHC, qRT-PCR, histomorphometry	-	2
III	8	yes	WT, 12w	Healed extraction socket	1.0	10	Histology, IHC, qRT-PCR, histomorphometry	-	2
III	8	yes	WT, 12w	Healed extraction socket	1.0	14	Histology, IHC, qRT-PCR, histomorphometry	-	2
I	8	yes	WT, 12w	Edentulous ridge	0.3	2	Histology, IHC, qRT-PCR, histomorphometry	-	2
I	8	yes	WT, 12w	Edentulous ridge	0.3	4	Histology, IHC, qRT-PCR, histomorphometry	-	SFig. 3
I	8	yes	WT, 12w	Edentulous ridge	0.3	7	Histology, IHC, qRT-PCR, histomorphometry	-	SFig. 3
I	8	yes	WT, 12w	Edentulous ridge	0.3	10	Histology, IHC, qRT-PCR, histomorphometry	-	2
I	8	yes	WT, 12w	Edentulous ridge	0.3	14	Histology, IHC, qRT-PCR, histomorphometry	-	2
III	8	yes	WT, 12w	Healed extraction	0.65	4	Histology, IHC, qRT-PCR,	0.6	3

				socket			histomorphometry		
III	8	yes	WT, 12w	Healed extraction socket	0.65	7	Histology, IHC, qRT-PCR, histomorphometry	0.6	3
III	8	yes	WT, 12w	Healed extraction socket	0.65	14	Histology, IHC, qRT-PCR, histomorphometry	0.6	3
I	8	yes	WT, 12w	Edentulous ridge	0.65	4	Histology, IHC, qRT-PCR, histomorphometry	0.6	3
I	8	yes	WT, 12w	Edentulous ridge	0.65	7	Histology, IHC, qRT-PCR, histomorphometry	0.6	3
I	8	yes	WT, 12w	Edentulous ridge	0.65	14	Histology, IHC, qRT-PCR, histomorphometry	0.6	3
I	6	no	<i>Axin2</i> ^{LacZ/+} 12w	Edentulous ridge	-	Skeletally mature adult	Histology, IHC, qRT-PCR	-	4
I	6	no	<i>Axin2</i> ^{CreERT2/+;mTmG/+} 12w	Edentulous ridge	-	Skeletally mature adult	Histology, IHC	-	4
III	6	no	<i>Axin2</i> ^{LacZ/+} 12w	Alveolar bone	-	Skeletally mature adult	Histology, IHC, qRT-PCR	-	4
III	6	no	<i>Axin2</i> ^{CreERT2/+;mTmG/+} 12w	Alveolar bone	-	Skeletally mature adult	Histology, IHC	-	4

Appendix Table 2. Methods of tissue preparation and analysis

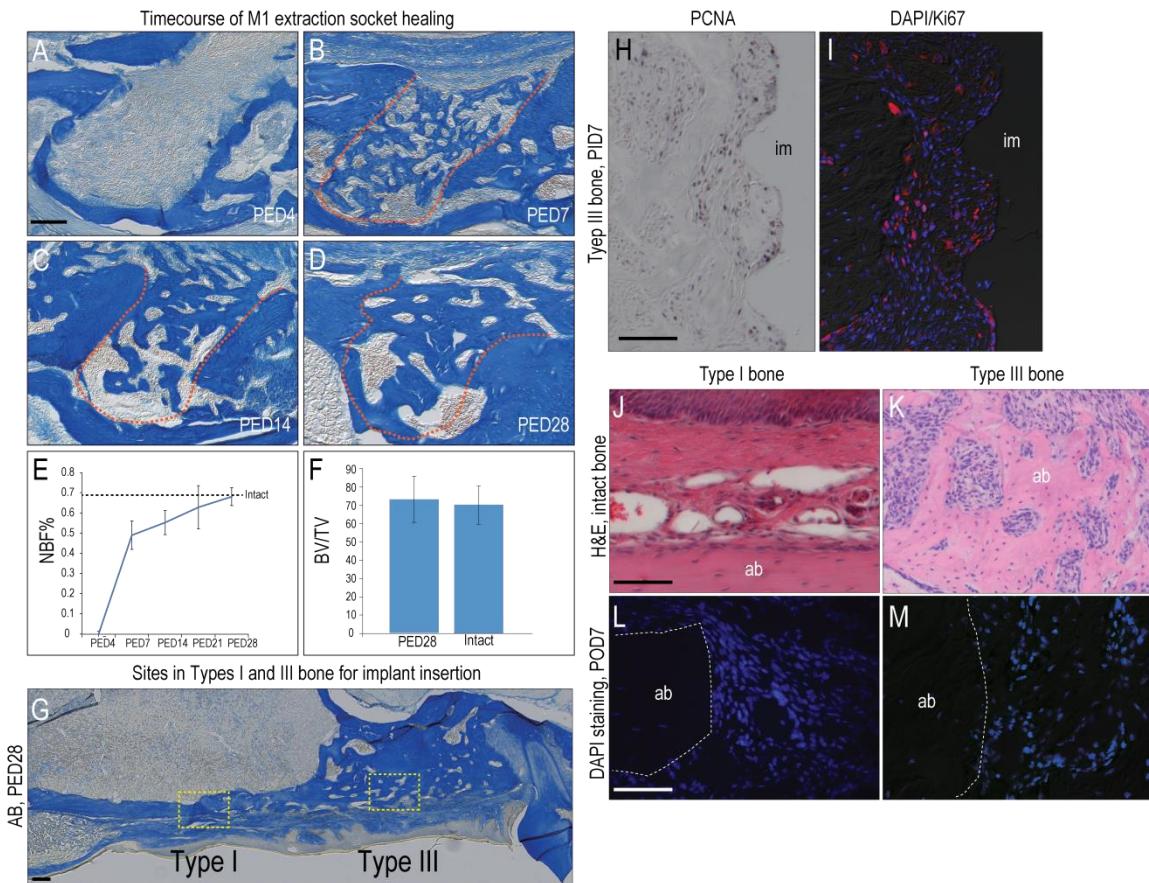
Chemistry/action and source	Concentration	Purpose	Reference
Paraformaldehyde (Sigma Aldrich)	4%	fixation	-
phosphate buffered saline (Fischer)	1x	rinsing	-
EDTA (Fischer)	19%	demineralization	-
Ethanol (Goldshield)	100-0%	dehydration	-
Xylene (Fischer)	100, 50%	Removal of ethanol	-
Paraffin (Fischer)	100%	Embedding for sectioning	-
Movat's pentachrome	-	Tissue analysis	(Lim et al. 2014a)
Aniline blue (Acros)	-	Osteoid matrix	(Lim et al. 2014a)
Picosirius red (Pfaltz and Bauer)	-	Collagen matrix	(Lim et al. 2014a)
Alkaline phosphatase activity (Roche)	-	Mineralization	(Minear et al. 2010)
Tartrate-resistant acid phosphatase (Sigma Aldrich)	-	Osteoclast activity	(Minear et al. 2010)
Xgal staining (Invitrogen)	-	Detection of beta galactosidase activity	(Minear et al. 2010)
anti-PCNA (Abcam)	1:10000	Cell proliferation	(Kubben et al. 1994)
anti-Osterix (Abcam)	1:500	Osteogenic lineage commitment	(Cao et al. 2005)
anti-Runx2 (Abcam)	1:100	Osteogenic lineage commitment	(Komori 2010)
anti-β-galactosidase (Abcam)	1:1000	Detection of LacZ gene product	(Juers et al. 2012)
anti-biotinylated GFP (Cell Signaling Tech)	1:500	Detection of GFP	-
Axin2 primer sequences	forward	[TCATTTCCGAGAACCCACCGC]	-
	reverse	[GCTCCAGTTTCAGTTCTCCAGCC]	-
Osterix	forward	[GGAGACCTTGCTCGTAGATTTC]	-
	reverse	[GGGATCTTAGTGACTGCCAAC]	-
GAPDH	forward	[ACCCAGAAGACTGTGGATGG]	-
	reverse	[GGATGCAGGGATGATGTTCT]	-



Appendix Fig. 1. Multiscale analyses of Type I and Type III bones.

(A) Quantification of osteocyte density and morphology in Type I and III bone. Representative tissue sections stained with Picosirius red then visualized under polarized light to illustrate (B) the lamellar organization of collagen in Type I bone versus the (C) basket-weave pattern of collagen organization in Type III bone. DAPI staining of cell nuclei in (D) Type I versus (E) Type III bone. PCNA immunostaining in (F) the gingiva adjacent to the edentulous ridge and (G)

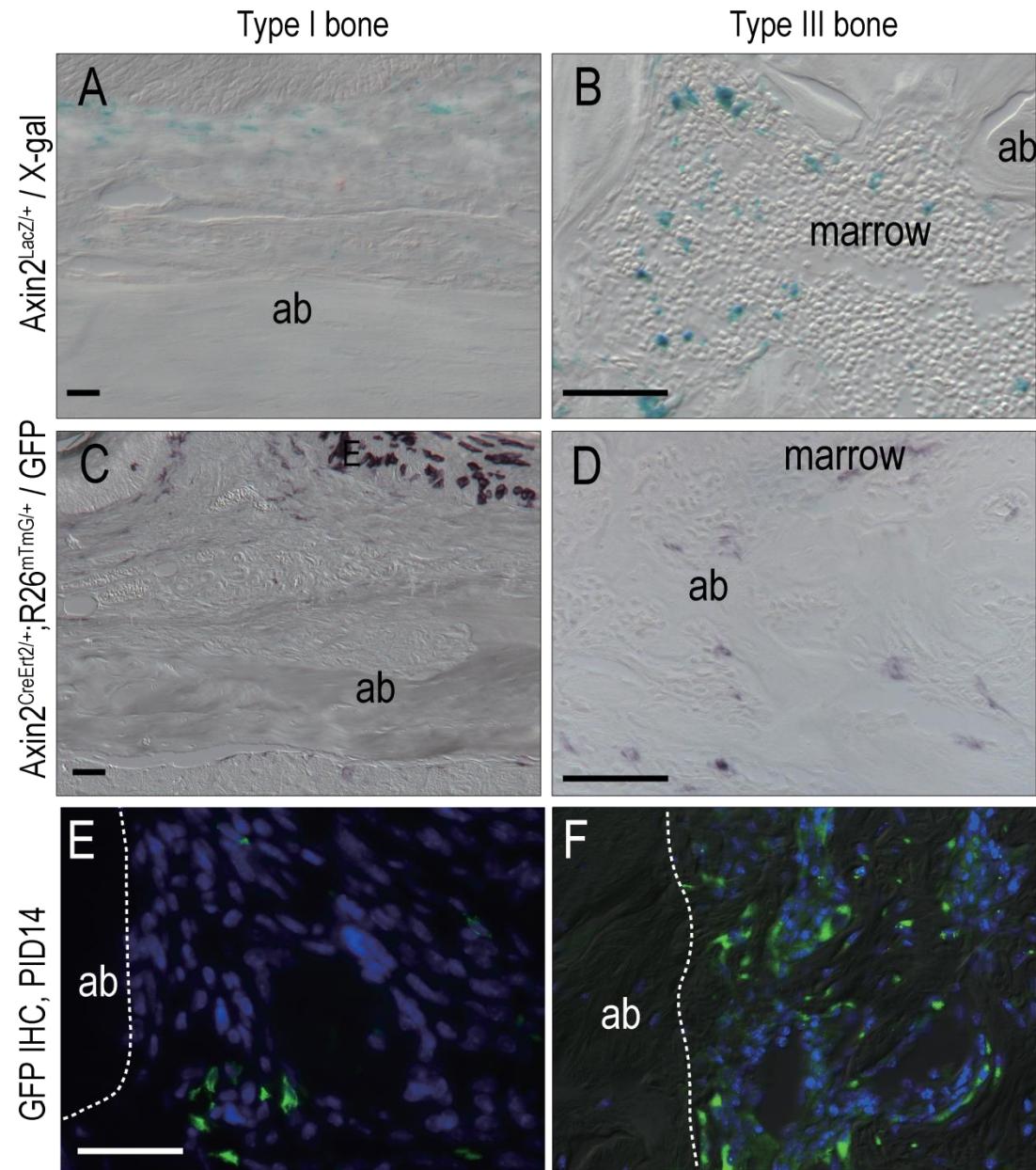
in the marrow cavities of alveolar bone. Ki67 immunostaining in (H) Type I and (I) Type III bone. ALP activity in (J) the periosteum and endosteum of Type I bones. (K) In Type III bones, ALP activity is also abundant in the vascular lacunae. CD31 immunostaining in (L) Type I and (M) Type III bone. Scale bar = 50 μ m for all panels.



Appendix Fig. 2. Extraction socket healing, sites for implant placement, cell proliferation in interfacial tissues and counter-staining of Types I and III bone.

Representative tissue sections stained with Aniline blue to identify new osteoid matrix in the healing M1 extraction socket on (A) PED4, (B) PED7, (C) PED14, and (D) PED28. (E) Quantification of new bone formation (NBF) at time points indicated. (F) BV/TV of the healed extraction socket on PED28 compared to the BV/TV of intact pristine maxillary bone. (G) Aniline blue staining of Type I and III bone sites prior to implant placement. (H) On PID7, PCNA immunostaining and (I) Ki67 immunostaining overlaid with DAPI to visualize mitotically active cells in the interfacial region around implants placed in Type III bone. H&E staining to identify distribution of cells in the periosteum of intact (J) Type I and (K) Type III bone. DAPI staining to identify cell nuclei in an osteotomy site in (L) Type I versus (M) Type III bone.

Abbreviations: ab, alveolar bone; PED, post-extraction day; POD, post-osteotomy day; PID, post-implant day. Scale bar = 50 μ m for all panels.



Appendix Fig. 3. Xgal and GFP staining to identify Wnt responsive cells in Type I and III bone, and GFP immunostaining to identify Wnt responsive progeny during osseointegration of implants in Type I and III bone.

Representative tissue sections from *Axin2*^{LacZ/+} mice, stained with Xgal to detect Wnt responsive cells in the periosteum of (A) Type I bone and (B) in the marrow spaces of Type III bone.

Representative tissue sections from $Axin2^{CreERT2/+};R26R^{mTmG/+}$ mice, immunostained to detect GFP in the periosteum of (C) Type I bone and (D) in the marrow spaces of Type III bone. GFP immunostaining to detect Wnt responsive progeny in interfacial tissues on PID14 in (E) Type I and (F) Type III bone. Abbreviations: ab, alveolar bone; IHC, immunohistochemistry; PID, post-implant day. Scale bar = 50 μ m for all panels.